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Characterization of melanoidins derived from Brewers' spent grain: New insights into their structure and antioxidant activity

Running title: Structural characterization of melanoidins

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Abstract

Melanoidins, formed at the final stages of Maillard reaction (MR) present important physiological activities, but their structure and reaction pathways are largely unknown in real food systems. In the present work, these bioactive compounds derived from Brewers' spent grain (BSG) with different roasting degrees were analyzed.

Results showed that BSG could be considered a more important source of bioavailable antioxidants than what it was reported in previous studies. During MR development, proteinic structures are partially lost while a carbohydrate skeleton is formed. The crystallinity is reduced and the structure sets into a conformation with a peak centred at 20°(2θ), stable with the increasing thermal treatment. While MR continues the development of browning compounds increases, the antioxidant activity rises and phenolic compounds are bind to the melanoidin structure ($P \leq 0.05$). Moreover, FTIR analysis showed a dominance of α-glyosidic linkage and the presence of furanose rings in the melanoidins structure.

Keywords

Brewery waste- Brewers' spent grain- Melanoidins- Structure analysis- Antioxidant activity

Introduction

Brewers' spent grain (BSG) is the major by-product of the brewing industry. The average annual global production of BSG is estimated to be 39 million tonnes and it mostly is used as a low-value animal feed. Nonetheless, BSG is an important source of bioactive compounds. It contains a high amount of antioxidant phenolic compounds such as hydroxycinnamic acids ferulic, and p-coumaric acids (Lynch *et al.*, 2013). Moreover, recent research has indicated that, as a result of barley grain roasting, BSG also contains important amounts of Maillard reaction products (MRP) (Connolly *et al.*, 2013; Piggott *et al.*, 2014).

The Maillard reaction (MR) is a type of non-enzymatic browning central to food chemistry because it leads to changes in flavour, taste and texture during processing (Bruhns *et al.*, 2019). Besides, in recent years, intertest has been focused on the biological activities of high molecular weight (HMW) products (better known as melanoidins) formed at the final stages of the MR. Melanoidins, exhibit an important antioxidant, antihypertensive and prebiotic activity that has been described in real food

systems (Wang *et al.*, 2011). Thus, the interest in melanoidins has increased dramatically in the last decade.

Melanoidins are coloured, nitrogen-containing polymeric compounds. However, their structure and reaction pathways are largely unknown, mainly because the complex array of melanoidins is strongly dependent on the type of food, the technological conditions of the reaction, pH and the composition of the reagents involved (Wang *et al.*, 2011). A current study performed by Mohsin *et al.* (2020) has tackled the different chemical pathways for melanoidins formed from glucose, alanine and fructosylalanine. While Bruhns *et al.* (2019) evaluated the formation mechanism involved in the MR of D-glucose and γ-aminobutyric acid at low water content. However, these analyses were performed using over-simplistic model systems, and little work has been done on real food systems.

A recent research performed by Yang *et al.* (2019) evaluated the content and preliminary structure of melanoidins isolated from the spent grains of a Chinese liquor. Nonetheless, it would be of major significance perform these analyses on BSG, as beer is the fifth most consumed beverage in the world (Olajire, 2020). On the other hand, although previous studies performed by Piggott *et al.* (2014) and Carvalho *et al.* (2014) analyzed MRP on beer by products and barley, these analyses only considered the chemical aspects of these bioactive compounds and currently there is not a proper description of the structure and the physical characteristics of HMW MRP derived from BSG.

Considering the importance of melanoidins in the diet and their physiological effects, an accurate assessment of their structure is of striking significance. In the present work the physical characteristics of these MRP as well as their relationship with their molecular structure were detailed studied. Moreover, the present work provides new insights into the antioxidant activity of these macromolecules. This is the first approach to understand the molecular stabilization process involved in the development of MRP derived from BSG. Besides, it could be the starting point for further applications of MRP as potential functional ingredients and for the development of tailor-made melanoidins.

Materials and Methods

Materials

BSGs with different roasting levels (Pilsen, Caramel 60, Caramel 120 and Chocolate) were obtained in the laboratory under equivalent industrial conditions (Palmer, 2006). Sodium carbonate, sodium acetate and acetic acid were supplied by Biopack. DPPH reagent, gallic acid, Folin-Ciocalteu reagent, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox standard), ABTS reagent and potassium persulphate were purchased from Sigma-Aldrich, while TPTZ reagent was supplied by Fluka Chemicals. All these chemicals were of analytical grade.

In vitro digestion and antioxidant determination

The digestion process of BSG samples was performed according to Patrignani *et al.* (2019). The antioxidant capacity of the soluble fraction and the solid residue obtained was determined by three different methods: DPPH, ABTS and FRAP. All the determinations were performed at least in duplicate, and the results were expressed as μmol Trolox equivalents per mg of dried sample. The global antioxidant capacity of the samples was calculated as the sum of the antioxidant capacity of the two fractions obtained. A detailed description of the techniques used can be found in the Supporting Information of this manuscript.

Aqueous extraction of melanoidins from BSG and characterization

The extraction of melanoidins from BSG was performed in accordance with Piggott *et al.* (2014). A proper amount of BSG was subjected to aqueous extraction (1:15 dw/v) at 70 °C for 1 h. Then, samples were centrifuged 4190 g for 20 min, and dialysed for 4 h against water at 60 °C with constant stirring (four water changes). The HMW retentate (>12 KDa) was freeze-dried weighed and stored at -20 °C.

The isolated MRP were resuspended in an appropriate volume of distilled water (0.6 mg/mL) and the UV–Vis spectrum (400–700 nm) was determined in duplicate for each sample. Also, sugar and protein concentration was measured by the anthrone and Bradford methods respectively (Piermaria *et al.*, 2015).

Determination of antioxidant activity and total phenolic content in melanoidins

The total phenolic content (TPC) of melanoidins was determined at least in triplicate on the solution obtained after aqueous hydrolysis and extraction (see previous section). Briefly, 25 µL of the extract and 50 µL of Folin-Ciocalteu reagent were added to 2325 µL of distilled water. Then, 100 µL of a Na_2CO_3 solution (20 % prepared in NaOH 0.1 M) was added. After 90 min of reaction at room temperature (25 °C) the absorbance was measured at 750 nm, and results were expressed as mg of gallic acid per g of dry melanoidin (Hasperué *et al.*, 2015). The antioxidant activity of MRP was determined by DPPH and FRAP methods as previously described.

Differential scanning calorimetry (DSC) analysis on melanoidins

A Q100 differential scanning calorimeter (TA Instruments, USA) was used to study the thermal transitions of BSG melanoidins. Freeze-dried samples (1.5 mg) were heated from 20 °C to 300 °C (10 °C/min) in sealed pans (Manzocco *et al.*, 1999). The enthalpy and peak temperature were obtained from the thermograms. Assays were performed at least in duplicate.

X-ray diffraction (XRD)

X-ray diffraction analysis was performed using a Philips diffractometer-APD PW 1710. Samples were irradiated with Cu Ka radiation (1.542 Å) using 30 mA and 40 kV. The diffraction patterns were obtained in the range of 3–50°(2θ) at a scan speed of 0.04 s^{-1} (Kang, 2016)

Fourier transform infrared spectroscopy (FTIR)

The infrared spectra of the samples were recorded between 4000 and 650 cm^{-1} at a resolution of 4 cm^{-1} using a Nicolet iS10 spectrometer (Thermo Scientific, USA) mounted with an attenuated total reflectance (ATR) accessory. Sixty-four scans were co-added for

each spectrum to improve the signal-to-noise ratio. The powder was directly placed on a single reflection diamond ATR accesory and pressed to have a good contact between the crystal and sample. Spectra processing was performed using the software EZ Omnic (Thermo Electron Corporation, USA).

Statistical analysis

Results were statistically evaluated by analysis of variance (ANOVA) at a 0.05 significance level. The least significant differences (LSD) were determined by comparing the means at a 95% confidence level using the Fisher's test (InfoStat, 2012;UNC, Argentina).

Results and Discussion

Antioxidant activity of BSG

In the present work, the release of bioaccessible antioxidant compounds was determined through a standardized static *in vitro* digestion method. Besides, the antioxidant capacity of the non-bioaccessible fraction was also considered as it may protect epithelial cells in the colon (Patrignani *et al.*, 2019). Because of the complexity of oxidative reactions, a combination of antioxidant tests was used in order to obtain accurate results. FRAP and DPPH showed that the antioxidant capacity significantly increased with the roasting degree of the BSG ($P \leq 0.05$). According to the results, the highest total antioxidant activity was found in Chocolate BSG (0.2 µmol/mg and 0.03 µmol/mg according to FRAP and DPPH analyses respectively) (Fig. 1). Other authors had previously determined the antioxidant capacity of BSG, but their results were significantly lower Connolly *et al.* (2013) indicated that the highest content of antioxidants in BSG determined by FRAP was 0.02 µmol/mg, while Ivanova *et al.* (2017) reported 0.006 µmol/mg and 0.005 µmol/mg according to FRAP and DPPH assays. Although ABTS assay did not indicate any significant difference among the samples ($P > 0.05$), the antioxidant capacity determined with this method was also significantly higher than previous studies. Socaci *et al.* (2018) found that the antioxidant capacity of BSG by ABTS was 6.10^{-6} µmol/mg; in

contrast, our results showed that the antioxidant content of BSG was 0.15 μmol/mg when an *in vitro* digestion process was used. The low values of antioxidant capacity found in previous works could be due to the chemical extraction used to release the bioactive compounds from the food matrix. Delgado-Andrade *et al.* (2010) indicated that the use of an *in vitro* digestion procedure is a critical step that allows the release of a large amount of antioxidant compounds and results can be considered more nutritionally relevant than the values determined in organic extracts.

Moreover, even though the BSG has a high proportion of fibre, most of its antioxidants could be successfully released from the food matrix by the digestive enzymes. This indicates that most antioxidants from BSG are bioaccessible and potentially bioavailable depending on their absorption through the intestinal wall (Patrignani *et al.*, 2019). It could be concluded that, although the use of different solvents to extract antioxidants in cereal-based foods is a common practice, it may be incomplete or inadequate for BSG (Delgado-Andrade *et al.*, 2010). According to our results, BSG could be considered a more important source of bioavailable antioxidants than what it was previously reported.

Composition and antioxidant capacity of isolated BSG melanoidins

Although melanoidins are one of the most important antioxidant compounds in BSG, their structure is largely unknown (Piggott *et al.*, 2014). The complexity of MR, in addition to the difficulty in purifying and identifying the melanoidins, makes their analysis challenging (Pastoriza and Rufián-Henares, 2014).

In the present work, the proportion of melanoidins in the BSG ranged from 6%-10% with no significant difference among the samples ($P > 0.05$) (data not shown). These values compare well with those reported by Pastoriza and Rufián-Henares (2014) who indicated that the melanoidin content in Pilsen and Black beer was 8% and 15% respectively. Moreover, according to the results displayed in Table 1, BSG isolated melanoidins had a low protein content and a high proportion of carbohydrates (73%-85%) (Table 1). This result is also in good agreement with Pastoriza and Rufián-Henares (2014), who found that the protein proportion in melanoidins isolated from beer was 72 ±

2%. Therefore, it could be considered that melanoidins extracted from BSG have similar characteristics to melanoidins isolated from beer.

On the other hand, as detailed in Table 1, the protein content of the melanoidins was associated with the roasting degree of the BSG ($P \leq 0.05$). The highest amount of protein was found in Pilsen BSG melanoidin (4.81 %), while the other samples showed a significantly lower proportion of this component (0.6%-1%) ($P \leq 0.05$). Besides, there was a low presence of phenolic compounds within the structure of melanoidins. This was previously reported by Piggott *et al.* (2014) in MRP derived from BSG, who supported the idea that these MRP have a carbohydrate-phenol based structure. Moreover, according to De La Cruz *et al.* (2019), during the roasting process phenolic compounds could bind to the initial melanoidin skeleton composed of carbohydrates and proteins. Phenolic compounds, are well known because of their strong antioxidant activity, and it is probable that they could act together with the melanoidins in the gastrointestinal tract (Saura-Calixto, 2011). It is therefore possible that the biological functions of MRP might be associated, with the presence of phenolic compounds in their structure.

Finally, the antioxidant capacity of the isolated melanoidins was determined by DPPH and FRAP (Table 1). Consistent with results obtained after *in vitro* digestion of the BSG (see previous section), the lowest antioxidant capacity was found in melanoidins extracted from Pilsen BSG, while roasted samples presented a higher antioxidant activity ($P \leq 0.05$).

UV–Vis spectrum of melanoidins

The absorption spectra of the different melanoidins were recorded in the four samples analysed. Absorbance at 294 nm is associated with the presence of early and low molecular weight MRP, while the development of high molecular weight MRP can be followed by an increase in the absorbance at 420 nm. Therefore, these values can be used as arbitrary measurements of melanoidin presence (Patrignani *et al.*, 2019). As illustrated in Fig. 2, the Abs420 and Abs294 of melanoidins increased with the roasting intensity of the BSG. Therefore, it could be established that the browning level of MRP in the samples was in the following order: Pilsen < Caramel 60≈Caramel 120 < Chocolate.

The absorbance parameters of the isolated melanoidins in pale and black beer (K_{420}=0.33 L/g.cm and K_{420}=0.52 L/g.cm respectively) determined by Pastoriza and Rufián-Henares (2014) were consistent with the ones found in the present work in Pilsen (K_{420}=0.33 L/g.cm) and Chocolate (K_{420}=0.66 L/g.cm) BSG melanoidins. On the other hand, the high absorbance found in far UV confirms the presence of peptide bonds in melanoidin structure (Patrignani *et al.*, 2019).

DSC analysis of melanoidins

DSC analysis is a widely accepted methodology to study the thermal behaviour of food. However, few researchers have attempted to detect the changes in enthalpy involved in the MR by DSC and published data are scarce (Manzocco *et al.*, 1999). In the present work, an endothermal peak was observed in the thermograms of melanoidins extracted from BSG. The thermograms together with the enthalpy values (ΔH) are shown in Fig. 3. According to the results, the ΔH of the peak decreased with the roasting degree of the samples, suggesting that it may be related to the presence of proteinic structures that are lost because of the thermal treatment (Biliaderis, 1983). Moreover, the peak temperature also seemed to increase with the thermal treatment. These results are consistent with Manzocco *et al.* (1999) who studied the thermal changes of glucose-glycine model systems. According to their results the prolonged heating of the solution caused the progressive browning of the samples which was associated with a decrease in ΔH. Besides, their results showed that the temperature of the peak shifted from 120°C to 150°C. However, these analyses were performed in model systems, and currently there are no references available about the thermal behaviour of real systems such as melanoidins extracted from BSG.

Therefore, it could be concluded that DSC analysis could be a useful methodology to evaluate the development of MR. Nonetheless, future studies should be carried out on real food systems in order to expand our knowledge about the thermal behaviour of MRP.

X-ray diffraction patterns of melanoidins

The X-ray diffraction patterns were performed to examine the crystallinity of BSG melanoidins. According to the results displayed in Fig. 4, broad diffraction peaks were found and important differences among roasted and non-roasted samples were observed. Pilsen melanoidins presented a main peak at 12° (2θ) while other samples showed a peak centred at 20° (2θ). These differences are due to the different conformations in the chemical structure related to the evolution of MR (Khadidja *et al.*, 2017). Also, it can be observed that as the roasting degree increased, the crystallinity was reduced (Fig. 4). In good agreement, other authors have reported that the development of MR reduces the crystallinity of the reactants (Lin *et al.*, 2019).

Although Caramel 60 and 120 presented a lower development of MR than Chocolate melanoidins (see previous section), their diffraction patterns were extremely similar. Therefore, it could be speculated that during the development of MR, the crystallinity is reduced and the structure sets into a new and different conformation with a peak centred at 20°(2θ). This final conformation seems to be stable and it is not modified with the increasing thermal treatment.

FT-IR analysis

FTIR spectroscopy was used for the analysis of the derivatives formed during the non-enzymatic browning reaction (Fig. 5). The broad, intense stretching signal at 3308 cm^{-1} represents hydroxyl in water and amine groups (Borel *et al.*, 2018; Silbir and Goksungur, 2019). The signal around 2900 cm^{-1} can be related to the asymmetric stretch (nC–H) of $–CH_2$ groups and the corresponding symmetric stretch can be observed at 2855 cm^{-1}. This last signal at 2855 cm^{-1} is progressively lost from Pilsen to Chocolate, which indicates a structure modification when MR takes place.

The region between 1650 and 1540 cm^{-1} is related to amide I and amide II structures, including C=O and C-N stretching and N-H deformation. While the adsorption band around 1300-1200 cm^{-1} can be attributed to amide III structures, including C-N stretching and N-H deformation. Changes of amide I and amide II bands in Fig. 5 show that hydroxyl and amino groups were consumed during the heating process. In particular, the band at 1543 cm^{-1} showed a significant decrease in intensity when the roasting degree increased (Fig. 5 (a) to (d)). These results are in accordance with previous

studies performed by Khadidja *et al.* (2017) in aqueous model systems containing gelatin and sodium alginate. Their results suggested that during the cross-linking of the material, proteinic structures may be lost. This was also observed in our study during the development of BSG melanoidins, and it is in good line with results shown in Table 1 and Fig. 3, which indicated that the protein structure of melanoidins was significantly reduced with the roasting degree.

The region between 1180 and 953 cm-1 is related to saccharide bonds vibrational modes, such as C-C and C-O stretching, and C-H bending (Kim and Lee, 2009; Borel *et al.*, 2018). The absorption at 1148 cm^{-1} indicated a dominance of α-glycosidic linkage (Mutaillifu *et al.*, 2020). The increase of this band with the roasting degree may be associated with formation of a melanoidin skeleton with carbohydrate side chains. This is also supported with results displayed in Table 1, which show that the proportion of carbohydrates was higher in the samples with an important roasting degree.

On the other hand, the number of compounds associated with non-enzymatic browning, such as the Amadori compounds (C=O), Schiff base (C=N), and pyrazines (C–N) may increase during MR (Farhat *et al.*, 1998). As detailed in Fig. 5, the peak at 1077 cm^{-1} showed an increase in intensity in good line with the malt roasting degree, which suggests the presence of furanose rings in the melanoidin structure. These basic structural units in melanoidins have been reported in previous studies (Bruhns *et al.*, 2019).

Conclusions

BSG is an important source of bioaccessible melanoidins with high antioxidant activity. The current results indicate that during the roasting process melanoidin structure is modified, proteinic structures are significantly reduced and phenolic compounds bind to the increasing carbohydrate skeleton. The crystallinity is also reduced and the structure sets into a new and different conformation with a peak centred at 20°(2θ) that is stable with the increasing roasting process. While MR continues, absorbance of melanoidins increases together with the antioxidant activity. FTIR analysis showed a dominance of α-glyosidic linkage and the presence of furanose rings in BSG melanoidin structure.

Although the potential uses of melanoidins are still undervalued, the analysis of their structure is the first step to open new fields of application and give a better understanding of their physiological effects.

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Conflict of Interest

The authors declare having no conflict of interest.

Data Availability Statement

Data that support our findings are available upon request to the authors.

Ethical Guidelines

Ethical approval was not required for this research

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Annoted references

The references with asterisks are key because, among those cited and listed, these studies deal with important advances in the characterization of melanoidins.

Supporting Information

Additional Supporting Information may be found in the online version of this article

Table 1. Protein content, carbohydrate content, antioxidant capacity and total phenolic content of melanoidins extracted from different BSG (Pilsen, Caramel 60, Caramel 120, and Chocolate)

	Protein content (%)	Carbohydrate content (%)	TPC (%)	FRAP (μg Trolox/mg)	DPPH (μg Trolox/mg)
Melanoidin of Pilsen BSG	4.81 ± 0.69^{b}	72.8 ± 3.6^{a}	8.84 ± 0.78^{a}	19.28±0.04^{a}	1.43±0.62^{a}
Melanoidin of Caramel 60 BSG	1.00 ± 0.04^{a}	76.16 ± 3.32^{ab}	14.88 ± 2.50^{ab}	37.4±2.00^{b}	18.00±3.00^{c}
Melanoidin of Caramel 120 BSG	0.64 ± 0.19^{a}	86.62 ±11.08^{b}	18.11 ± 3.70^{b}	40.40±1.65^{b}	9.15±0.44^{b}
Melanoidin of Chocolate BSG	1.00 ± 0.25^{a}	84.8 ± 3.14^{b}	18.38 ± 0.74^{b}	51.34±1.13^{c}	8.80±2.60^{b}

Results are expressed as mean ± standard deviation. Values in the same column followed by different superscript letters are significantly different ($P \leq 0.05$).

Legends to Figures

Fig.1

Bioaccessible (soluble fraction), insoluble (solid residue) and total antioxidant activity after the in vitro digestion process of BSG with different roasting degree. Determinations were performed with FRAP(a), DPPH(b) or ABTS(c) assays. Different letters indicate significant differences between the different antioxidant fractions ($P \leq 0.05$). Results represent mean ± standard deviation.

Fig. 2

Absorption spectra of BSG melanoidins (0.6 mg/ml) (Pilsen, Caramel 60, Caramel 120 and Chocolate). Results represent mean ± standard deviation.

Fig. 3

DSC curve of melanoidins isolated from Pilsen, Caramel 60, Caramel 120 and chocolate BSG.

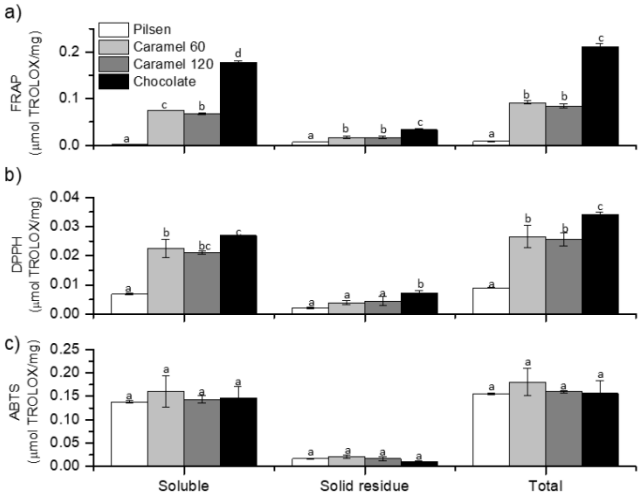
Fig. 4

X-ray diffractograms of melanoidins isolated from Pilsen, Caramel 60, Caramel 120 and chocolate BSG.

Fig. 5

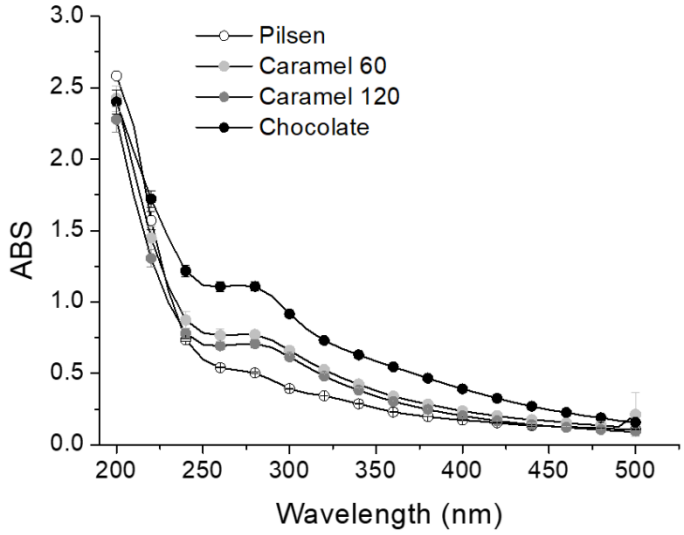
FTIR spectra of melanoidins isolated from Pilsen (a), Caramel 60 (b), Caramel 120 (c) and chocolate BSG (d) in the 3800–600cm^{-1} region.

Fig. 1



ijfs_14653_f1.tif

Fig. 2



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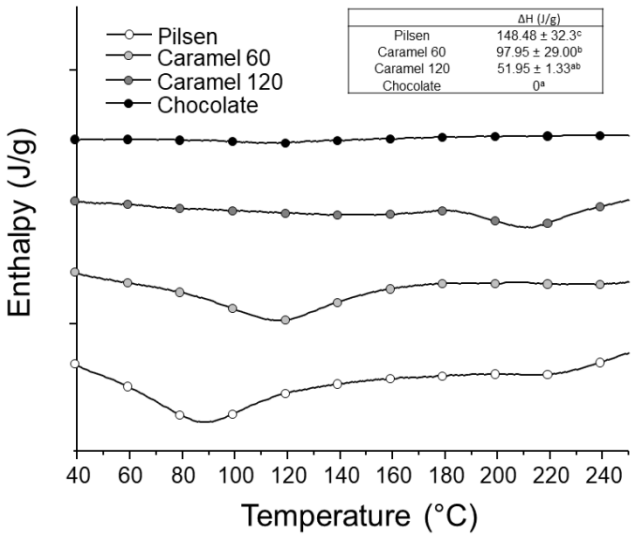

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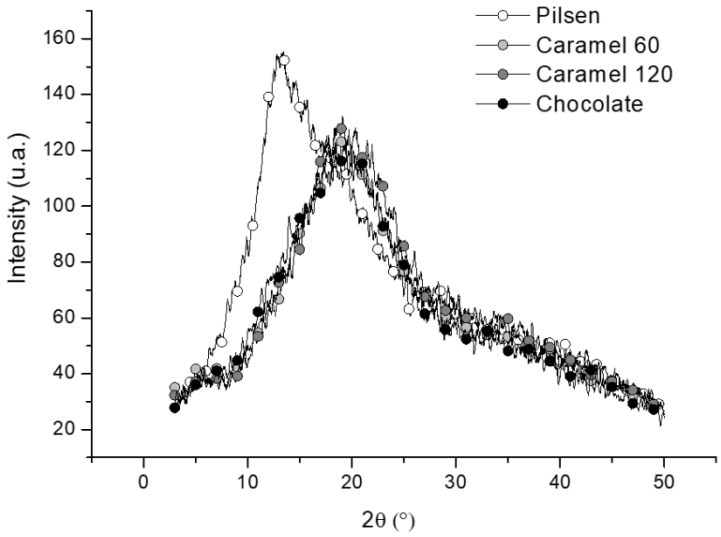
Fig. 4



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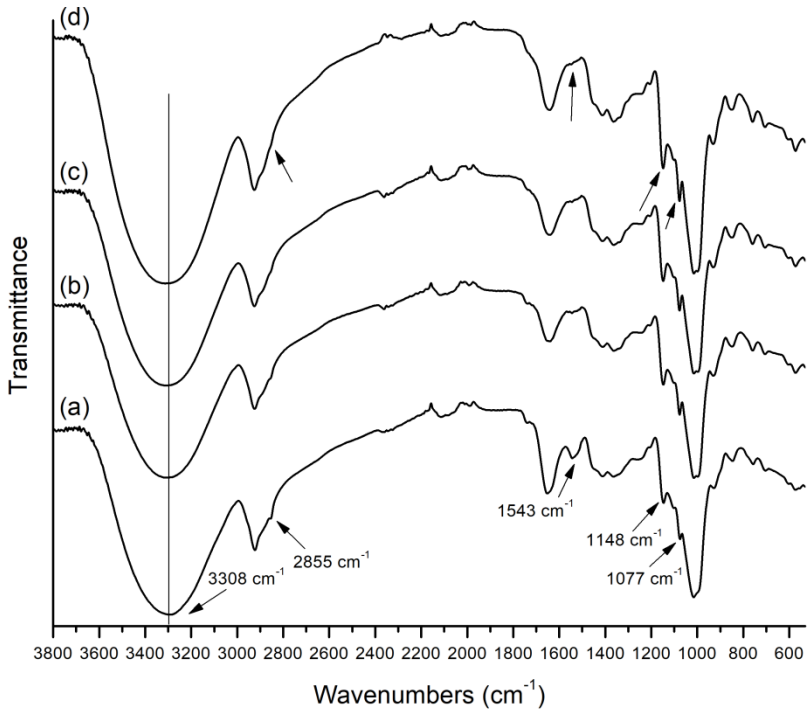

ijfs_14653_f5.tif